DESCRIPTION

TITLE OF THE INVENTION

GENE IMPROVING TEMPERATURE-TOLERANCE OF ACETIC ACID BACTERIUM, ACETIC ACID BACTERIUM BRED USING THE GENE AND PROCESS FOR PRODUCING VINEGAR USING THE ACETIC ACID BACTERIUM

Technical Field to Which the Invention Pertains

The present invention relates to a gene that encodes a protein having a function of enhancing temperature tolerance derived from a microorganism, a microorganism in which the copy number of the gene is amplified, particularly an acetic acid bacterium which belongs to the genus Acetobacter or the genus Gluconacetobacter, and a method of efficiently producing vinegar containing acetic acid at a high concentration by using such a microorganism.

Prior Art

In industrial vinegar production, acetic acid fermentation by microorganisms is utilized. Such microorganisms have alcohol oxidation ability and are generally called acetic acid bacteria. Among acetic acid bacteria, particularly acetic acid bacteria which belong to the genus Acetobacter or the genus Gluconacetobacter are widely utilized for industrial acetic acid fermentation.

In acetic acid fermentation, ethanol in a medium is oxidized and converted to acetic acid by acetic acid bacteria, and as a result, acetic acid accumulates directly in the medium. At this time, a large amount of heat of fermentation is generated, and as a result, the temperature in the fermentation liquid increases when it is left alone as it is. An optimal fermentation temperature for acetic acid bacteria is generally around 30°C, therefore, it is necessary to cool the fermentation liquid in order not to increase the temperature thereof, which results in needing energy for cooling. Accordingly, it is required in acetic acid fermentation that growth ability and fermentation ability do not decrease even at a higher temperature, i.e. development of acetic acid bacteria having strong temperature tolerance is required. It has been attempted as one of the means that temperature-tolerant acetic acid bacteria are searched by screening acetic acid bacteria with temperature tolerance from nature (see, non-patent literature 1, for instance).

However, there are few findings on temperature-tolerance genes of acetic acid bacteria, and it was desired to obtain a novel temperature-tolerance gene that encodes a protein having a function capable of improving the temperature tolerance of acetic acid bacteria on a practical level and to breed acetic acid bacteria having stronger temperature tolerance with the use of the obtained temperature-tolerance

gene.

Patent literature 1

Japanese Laid-Open Patent Application No. 60-9488

Patent literature 2

Japanese Patent Application No. 2003-350265

Non-patent literature 1

Agricultural and Biological Chemistry, vol. 44, p. 2901-2906, 1980

Non-patent literature 2

Trends in Genetics, vol. 5, p. 185-189, 1989

Non-patent literature 3

Applied and Environmental Microbiology, vol. 55, p. 171-176, 1989

Non-patent literature 4

Agricultural and Biological Chemistry, vol. 52, p. 3125-3129, 1988

Non-patent literature 5

Agricultural and Biological Chemistry, vol. 49, p. 2091-2097, 1985

Non-patent literature 6

Bioscience, Biotechnology and Biochemistry, vol. 58, p. 974-975, 1994

Non-patent literature 7

Cellulose, p. 153-158, 1989

Non-patent literature 8

Journal of Bacteriology, vol. 175, 6857-6866, 1993

Problem to be Solved by the Invention

As mentioned above, no example that has elucidated temperature tolerance of acetic acid bacteria on a genetic level and has succeeded in the development of practically usable acetic acid bacteria having high temperature tolerance has been reported heretofore. However, development of acetic acid bacteria superior in temperature tolerance would allow the performance of acetic acid fermentation at a higher temperature than conventionally and reduction of the cost of cooling. Therefore, the present inventor attempted again to elucidate the improvement of temperature tolerance of acetic acid bacteria on a genetic level.

As a result of consideration from various aspects, and from the view point that it was important to obtain a novel temperature-tolerance gene that encodes a protein having a function capable of improving temperature tolerance on a practical level and to breed acetic acid bacteria having stronger temperature tolerance with the use of the obtained temperature-tolerance gene, the present inventor has newly set novel technical tasks to provide a novel gene for improving temperature tolerance, which participates in temperature tolerance and is derived from a microorganism belonging to

acetic acid bacteria, and to provide a method of improving temperature tolerance of a microorganism by using the gene, particularly a method of improving temperature tolerance of a microorganism belonging to acetic acid bacteria, further a method of efficiently producing vinegar by using the acetic acid bacteria whose temperature tolerance was improved.

Means for Solving Problems

The present inventor hypothesized that a specific gene participating in temperature tolerance that does not exist in other microorganisms should exist in acetic acid bacteria capable of growing and fermenting even under a high temperature, and obtained a novel concept that the use of such a gene would allow the improvement of temperature tolerance of a microorganism more than before and the development of an efficient production method.

As for a method of obtaining a temperature-tolerance gene in the conventional methods, a method of screening a temperature-insensitive mutant was popular.

However, thinking that it was difficult to find a temperature-tolerance gene, which was industrially useful, by such a method, the present inventor has investigated another acquisition method. As a result, the inventor developed a method of obtaining a gene enabling acetic acid bacteria, which can generally grow only at around 37°C on an agar medium, to

grow under a temperature of 38°C by constructing a chromosomal DNA library of acetic acid bacteria, transforming acetic acid bacteria with this chromosomal DNA library, and screening the target gene.

According to the use of this method, the present inventor has succeeded for the first time in cloning DNA coding for a novel temperature-tolerance gene that has a function of enhancing temperature tolerance on a practical level from acetic acid bacteria belonging to the genus Gluconacetobacter practically used in producing vinegar.

The obtained temperature-tolerance gene showed homology with a group of proteins referred to as ceramide glucosyltransferase found in leguminous bacteria and the like, and it was presumed as a gene that encodes ceramide glucosyltransferase of acetic acid bacteria as a result of homology search on DDBJ/EMBL/Genbank.

However, the obtained ceramide glucosyltransferase gene of acetic acid bacteria had an extremely low homology with known ceramide glucosyltransferase genes found in other microorganisms such as leguminous bacteria. Therefore, it was found that, though it was similar to other ceramide glucosyltransferase genes to some extent, the obtained gene was a novel gene encoding a novel protein (it is sometimes referred to as protein GCS) specific to acetic acid bacteria.

In addition, the present inventor found that, in the case

where the transformant was cultured with aeration in the presence of ethanol, the temperature tolerance was remarkably improved, the final acetic acid concentration can be remarkably improved, etc., and further have succeeded in determination of the amino acid sequence of the protein and the nucleotide sequence of the DNA of the gene encoding the protein, thus the present invention has been completed.

Brief Description of the Drawings

Fig. 1

This is a schematic view showing a restriction enzyme map of a gene fragment (pG1) derived from Gluconacetobacter entanii cloned by using restriction enzymes SalI and KpnI, the location of GCS gene and the insert into pGCS and pGCS1.

Fig. 2

This is a figure showing the time course in acetic acid fermentation by the transformant in which the copy number of GCS gene was amplified.

Fig. 3

This is a figure showing the time course in acetic acid fermentation by the transformant in which the copy number of GCS gene was amplified.

Fig. 4

This is a figure showing the amino acid sequence (SEQ. ID No. 2) of the protein encoded by GCS gene.

Fig. 5

This is a figure showing primer 1.

Fig. 6

This is a figure showing primer 2.

Fig. 7

This is a figure showing the nucleotide sequence (SEQ. ID No. 1) of the present temperature-tolerance gene.

Fig. 8

This is a figure showing the construction of pGI18.

Fig. 9

This is a figure showing the nucleotide sequence (SEQ. ID No. 5) of pGI18.

Fig. 10

This is a figure showing the nucleotide sequence (SEQ. ID No. 5) of pGI18 continued from Fig. 9.

Fig. 11

This is a figure showing the nucleotide sequence (SEQ. ID No. 5) of pGI18 continued from Fig. 10.

Fig. 12

This is a figure showing primer A.

Fig. 13

This is a figure showing primer B.

Disclosure of the Invention

That is, the present invention provides the following

(1) to (10) as examples of the embodiment.

(1) A protein GCS shown in the following (A) or (B):

(A) a protein having an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing,

(B) a protein consisting of an amino acid sequence comprising substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing and having a function of enhancing temperature tolerance.

(2) A DNA of a novel gene encoding a protein shown in the following (A) or (B):

(A) a protein having an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing,

(B) a protein consisting of an amino acid sequence comprising substitution, deletion, insertion, addition, or inversion of one or several amino acids in an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing and having a function of enhancing temperature tolerance.

(3) The DNA of a gene described in the above-mentioned (2), which is a DNA shown in the following (a) or (b):

(a) a DNA that comprises a nucleotide sequence consisting of nucleotides 73 to 1251 within the nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing,

(b) a DNA that hybridizes with a probe comprising a nucleotide sequence consisting of nucleotides 73 to 1251 within

the nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing or a part thereof under a stringent condition, and encodes a protein having a function of enhancing temperature tolerance.

(4) A microorganism whose temperature tolerance is enhanced by amplifying the intracellular copy number of the DNA described in the above-mentioned (2) or (3).

(5) The microorganism described in the above-mentioned (4) characterized in that the microorganism is an acetic acid bacterium belonging to the genus Acetobacter or the genus Gluconacetobacter.

(6) A method of producing vinegar characterized by culturing a microorganism having alcohol oxidation ability among the microorganisms described in the above-mentioned (4) or (5) in a medium containing alcohol, whereby acetic acid is produced and accumulated in the medium even at a high culture temperature.

(7) A recombinant plasmid pUCGCS (FERM BP-8217) including at least the DNA described in the above-mentioned (2) or (3).

(8) A recombinant plasmid pG1 or pGCS which is obtained by inserting PCR amplified fragments containing at least a DNA fragment having a nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing (SalI-KpnI fragment) or a coding region thereof (nucleotides 73 to 1251), respectively, into, for

instance, an acetic acid bacteria-Escherichia coli shuttle vector pGI18 (see, patent literature 2, for instance) not into an Escherichia coli vector pT7Blue as in (7).

(9) A transformant which is obtained by introducing a recombinant plasmid pG1 or pGCS into Acetobacter aceti No. 1023 (FERM BP-2287).

(10) A transformant which is obtained by introducing a recombinant plasmid pG1 or pGCS into Acetobacter altoacetigenes MH-24 (FERM BP-491).

According to the present invention, tolerance against temperature can be provided and enhanced for a microorganism. Moreover, in a microorganism having alcohol oxidation ability, particularly in acetic acid bacteria, tolerance against temperature can be remarkably improved and the ability of efficiently accumulating acetic acid even under a high temperature can be provided.

Best Mode for Carrying Out the Invention

Hereunder, the present invention will be described in detail.

(1) The DNA of the present invention

The DNA of the present invention comprises a nucleotide sequence that can encode a protein having a function of improving temperature tolerance and comprising an amino acid sequence shown in SEQ. ID No. 2 in the sequence listing, and

it comprises an element for regulating the nucleotide sequence and a structural portion of the gene. In more detail, the present invention relates to a temperature-tolerance gene, and the temperature-tolerance gene means a gene participating in temperature tolerance and/or improvement of temperature tolerance. More specifically, the gene comprises at least one selected from at least (i) DNA having a nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing and (ii) a nucleotide sequence of a gene that is included in (i) and encodes GCS protein (GCS gene).

The DNA of the present invention can be prepared by a method known to those skilled in the art. For example, DNA shown in a specific nucleotide sequence herein can be prepared by, for example, shotgun cloning with the use of a genome of acetic acid bacteria as a starting material. At this time, each of the fragmented chromosomal DNA is ligated to an appropriate cloning vector such as a plasmid vector or a phage vector depending on its size or the like, and an appropriate host cell such as an acetic acid bacterium is transformed with the use of this vector by an appropriate method such as electroporation, whereby a clone library for cloning each of the chromosomal DNA fragments can be prepared.

Further, determination of the nucleotide sequence of each of the chromosomal DNA fragments obtained from such a clone library can be carried out in accordance with a known method

such as a chemical degradation method (Maxam-Gilbert method) or a dideoxy method.

Alternatively, the DNA of the present invention can be prepared by a chemical synthesis well-known to those skilled in the art or amplification by the PCR method with the use of a primer of the present invention based on the information of the nucleotide sequence of the DNA of the present invention or the amino acid sequence described herein.

For example, the DNA of the present invention can be obtained from chromosomal DNA of Gluconacetobacter entanii as follows. First, chromosomal DNA of Gluconacetobacter entanii, for example, Acetobacter altoacetigenes MH-24 strain (FERM BP-491) is obtained. Chromosomal DNA can be obtained by, for example, the method disclosed in Japanese Laid-Open Patent Application No. 60-9489.

Next, a chromosomal DNA library is prepared in order to isolate a gene for improving temperature tolerance from the obtained chromosomal DNA. To begin with, the chromosomal DNA is partially digested by a suitable restriction enzyme to obtain various chromosomal DNA fragment-mixtures. As the restriction enzyme, various kinds of restriction enzymes can be used, and the extent of cleavage is controlled by adjusting cleavage reaction time and the like depending on the used enzyme. For instance, chromosomal DNA is digested by the action of Sau3AI at a temperature of 30°C or higher, preferably 37°C, at

an enzyme concentration of 1 to 10 units/ml for various time periods (1 min to 2 h). Meanwhile, SalI and KpnI were used in the after-mentioned Examples.

Then, the digested fragments of chromosomal DNA are ligated to vector DNA that can replicate autonomously in acetic acid bacteria. Specifically, this vector DNA is completely digested by the action of a restriction enzyme which yields a terminal nucleotide sequence complementary to the restriction enzymes, SalI and KpnI, used in the digesting of the chromosomal DNA, for instance, SalI and KpnI, under the condition at a temperature of 37°C and an enzyme concentration of 1 to 100 units/ml for 1 h or longer.

The digested vector DNA is mixed with the chromosomal DNA fragment-mixture, then a target recombinant DNA (DNA library) can be obtained by the action of T_4 DNA ligase. Incidentally, the condition for the action of T_4 DNA ligase can be set, for instance, at a temperature of 4 to 16°C and an enzyme concentration of 1 to 100 units/ml for 1 h or longer, preferably 6 to 24 h.

Using the obtained recombinant DNA, an acetic acid bacterium, which can generally grow only at 37°C on an agar medium, for instance Acetobacter aceti No. 1023 strain (FERM BP-2287) is transformed, and cultured at 38°C. A DNA fragment including a temperature-tolerance gene can be obtained by inoculating a formed colony into a liquid medium for

cultivation, then recovering plasmids from the obtained bacterial cells.

As for the DNA of the present invention, DNA comprising a nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing is specifically exemplified, among which the nucleotide sequence consisting of nucleotides 73 to 1251 within the nucleotide sequence is a coding region.

As a result of homology search on DDBJ/EMBL/Genbank and SWISS-PROT/PIR about the nucleotide sequence shown in SEQ. ID No. 1 and the amino acid sequence shown in SEQ. ID No. 2 (Fig. 4: corresponding to nucleotides 73 to 1251), it was found that they showed 41% homology with ceramide glucosyltransferase gene of Mesorhizobium loti and 39% homology with ceramide glucosyltransferase gene of Agrobacterium tumefaciens on an amino acid sequence level, but each of the homologies was low (around 40%), therefore, it was apparent that this was a novel gene and different from the genes encoding these proteins. In addition, it has been totally unknown that the above-mentioned ceramide glucosyltransferase gene participates in temperature tolerance.

As the nucleotide sequence of the DNA of the present invention was revealed, for instance, the DNA of the present invention can also be obtained by polymerase chain reaction (PCR reaction) with the use of the genomic DNA of acetic acid bacteria as a template and an oligonucleotide synthesized based

on the nucleotide sequence as a primer, or by hybridization with the use of an oligonucleotide synthesized based on the nucleotide sequence as a probe, as well.

As for the synthesis of the oligonucleotide, it can be synthesized, for instance using commercially available various DNA synthesizers in the conventional manner. In addition, PCR reaction can be performed in the conventional manner using Thermal Cycler Gene Amp PCR System 9700 manufactured by Applied Biosystems and Taq DNA polymerase (manufactured by Takara Bio Inc.), KOD-Plus- (manufactured by TOYOBO Co., LTD.) and the like.

As for the DNA encoding a protein having a function of enhancing temperature tolerance of the present invention, it may be a DNA encoding a protein where one or several amino acids are deleted, substituted, inserted or added at one or several sites, as long as the function of enhancing temperature tolerance of the encoded protein is not impaired.

The DNA encoding a protein substantially identical to such a protein having a function of enhancing temperature tolerance can also be obtained by site directed mutation so that an amino acid at a specific site is deleted, substituted, inserted, added or inversed by site-directed mutagenesis, for instance. Further, the modified DNA as mentioned above can also be obtained by a conventionally known mutagenesis treatment.

In addition, since it is generally known that an amino acid sequence of a protein and a nucleotide sequence encoding the protein are slightly different among species, strains, mutants and variants, the DNA encoding a substantially identical protein can be obtained from general acetic acid bacteria, particularly from species, strains, mutants and variants of the genus Acetobacter or the genus Gluconacetobacter.

Specifically, DNA encoding a protein substantially identical to the protein can be obtained from acetic acid bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter, acetic acid bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter treated for mutagenesis, or natural mutants or variants thereof, by for instance, isolating DNA, which hybridizes under a stringent condition with DNA comprising a nucleotide sequence consisting of nucleotides 73 to 1251 within the nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing and encodes a protein having a function of enhancing temperature tolerance.

The stringent condition here is a condition in which so-called a specific hybrid is formed while a non-specific hybrid is not formed. Though it is difficult to quantify this condition clearly, if one example is taken, a condition in which DNAs having high homology, for instance DNAs having homology of 70% or more hybridize, while DNAs having homology lower than

this do not hybridize, or a condition in which general washing for hybridization is performed, for instance the washing is performed at a salt concentration equivalent to 1 x SSC and 0.1% SDS at 60°C, can be exemplified.

The confirmation of having a function of enhancing temperature tolerance can be performed by, for instance, transforming Acetobacter aceti No. 1023 strain (FERM BP-2287), which can generally grow only at around 37°C on an agar medium, with a target DNA, and investigating whether or not it can grow at 38°C as explained in the after-mentioned Examples.

(2) The acetic acid bacteria of the present invention

The acetic acid bacteria of the present invention mean bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter, or such bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter whose temperature tolerance is enhanced.

As for the bacteria belonging to the genus Acetobacter, Acetobacter aceti can be cited specifically, and for instance, Acetobacter aceti No. 1023 strain (deposited as FERM BP-2287 with International Patent Organism Depositary), Acetobacter aceti subsp. xylinum IFO 3288 strain and Acetobacter aceti IFO 3283 strain can be specifically exemplified.

Further, as for the bacteria belonging to the genus Gluconacetobacter, for instance, Gluconacetobacter europaeus

DSM 6160 and Gluconacetobacter entanii can be cited, and for instance, Acetobacter altoacetigenes MH-24 strain (deposited as FERM BP-491 with International Patent Organism Depositary) can be specifically exemplified.

Temperature tolerance can be enhanced, for instance, by amplifying the intracellular copy number of a temperature-tolerance gene, or by transforming bacteria belonging to the genus Acetobacter with the use of recombinant DNA obtained by ligating a DNA fragment containing a structural gene of the gene to a promoter sequence that functions efficiently in the bacteria belonging to the genus Acetobacter.

In addition, temperature tolerance can also be enhanced by replacing the promoter sequence of the gene on chromosomal DNA with another promoter sequence functioning efficiently in bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter, for instance a promoter sequence for alcohol dehydrogenase from acetic acid bacteria (see non-patent literature 8, for instance) or a promoter sequence derived from a microorganism other than acetic acid bacteria including a promoter for each gene such as an ampicillin-resistance gene for plasmid pBR322, a kanamycin-resistance gene for plasmid pACYC177, a chloramphenicol-resistance gene for plasmid pACYC184, a beta-galactosidase gene from Escherichia coli.

Amplification of the intracellular copy number of the

gene can be conducted by introducing a multi-copy vector retaining the gene into cells of bacteria belonging to the genus Acetobacter, i.e. it can be conducted by introducing a plasmid, transposon or the like retaining the gene into cells of bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter.

As for the multi-copy vector, a plasmid, transposon and the like can be exemplified. As for the plasmid, pMV24 (see non-patent literature 3, for instance), pGI18 (see non-patent literature 2, for instance), pUF106 (see non-patent literature 7, for instance), pTA5001 (A), pTA5001 (B) (see non-patent literature 1, for instance) and the like can be exemplified, and pMVL1 which is a chromosome-integrative type vector (see non-patent literature 4, for instance) can also be cited. Further, as for the transposon, Mu, IS1452 and the like can be exemplified.

Introduction of DNA into acetic acid bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter can be performed by the calcium chloride method (see non-patent literature 5, for instance), the electroporation method (see non-patent literature 6, for instance) or the like.

Enhancement of temperature tolerance in acetic acid bacteria belonging to the genus Acetobacter or the genus Gluconacetobacter having alcohol oxidation ability in the above-described manner enables increase of the production

efficiency of acetic acid.

(3) Method of producing vinegar

The method of producing vinegar of the present invention is characterized by culturing a microorganism which is a bacterium belonging to the genus Acetobacter or the genus Gluconacetobacter selectively enhanced its temperature tolerance by amplification of the copy number of a temperature-tolerance gene and which has alcohol oxidation ability in a medium containing alcohol, whereby acetic acid is produced and accumulated in the medium.

This method may be performed in the same manner as the conventional method of fermentation by acetic acid bacteria. The medium containing alcohol means a medium containing alcohol such as ethanol, carbon source, nitrogen source, inorganic substance and the like, and the one having a similar composition to that of a medium used in so-called acetic acid fermentation can be used. It may be either synthetic medium or natural medium as long as it contains a suitable amount of nutrition required by the used bacteria strain for its growth as needed. As for the carbon source, various kinds of carbohydrates such as glucose and sucrose and various kinds of organic acids can be used. As for the nitrogen source, natural nitrogen sources such as peptone, a degradation product of the fermentation bacteria and the like can be used.

In addition, the culture can be carried out in accordance with the usual methods such as static culture, shaking culture and aeration-agitation culture under aerobic condition. The culture temperature is 20 to 40°C, preferably 25 to 35°C and can be generally set at 30°C. The pH of the medium is generally within the range of 2.5 to 7.0, preferably within the range of 2.7 to 6.5, and it can be adjusted with various kinds of acids, bases, buffers and the like as needed.

As mentioned above, in the present invention, by cultivation in a medium containing alcohol, acetic acid can be produced and accumulated in the medium. Generally, by cultivation of 1 to 21 days, acetic acid is produced and accumulated at higher concentration in the medium.

(4) Embodiment of the present invention

A recombinant plasmid pUCGCS which are made by inserting the ORF according to the present invention (nucleotides 73-1251 in SEQ. ID No. 1: GCS gene) or a part of the temperature-tolerance gene (SEQ. ID No. 1) containing the ORF into an Escherichia coli vector (multi-copy vector) pT7Blue (manufactured by Novagen Co.) has been deposited as FERM BP-8217 with International Patent Organism Depositary, so that the DNA of the gene according to the present invention can be obtained without difficulty, and a person skilled in the art would easily carry out the present invention. In addition,

if it is desired, by using this recombinant plasmid, the ORF according to the present invention or a temperature- tolerance gene containing the ORF is transferred to a vector capable of replicating autonomously in acetic acid bacteria, and the vector is introduced into acetic acid bacteria and the acetic acid bacteria is cultured, whereby acetic acid fermentation can be carried out even under a high temperature condition and the cost for cooling can be reduced.

Further, as mentioned above and as it is also apparent from the after-mentioned Examples, the deposit of the source of the gene for enhancing temperature tolerance, the nucleotide sequence of the gene, the amino acid sequence of the protein corresponding to the gene, the embodiment of PCR, the preparation of a plasmid vector and a recombinant plasmid, the deposit of host bacteria and the like have been elucidated, and each of them can be obtained, operated and processed easily, therefore, if each operation and treatment is performed with reference to Examples, the target temperature-tolerant transformant can be obtained. The use of the transformant enables to produce acetic acid even under a high temperature condition.

Hereunder, the present invention will be explained more specifically with reference to Examples

Examples

(Example 1) Cloning of temperature-tolerance gene from Gluconacetobacter entanii and determination of the nucleotide sequence and the amino acid sequence.

(1) Generation of chromosomal DNA library

Acetobacter altoacetigenes MH-24 strain (FERM BP-491), which is one strain of Gluconacetobacter entanii, was cultured with shaking at 30°C for 240 to 336 h in YPG medium (containing 3% glucose, 0.5% yeast extract and 0.2% polypeptone) to which 6% acetic acid and 4% ethanol were added. After the cultivation, the culture medium was centrifuged (7,500 x g, 10 min) and bacterial cells were obtained. Chromosomal DNA was prepared from the obtained bacterial cells by the method disclosed in Japanese Laid-Open Patent Application No. 60-9489.

The obtained chromosomal DNA as mentioned above and the Escherichia coli-acetic acid bacteria shuttle vector pMV24 were digested with restriction enzymes SalI and KpnI (manufactured by Takara Bio Inc). These DNA in adequate amounts were mixed and ligated by using a ligation kit (TaKaRa DNA Ligation Kit Ver. 2, manufactured by Takara Bio Inc.), whereby a chromosomal DNA library of Acetobacter altoacetigenes MH-24 was constructed.

(2) Cloning of temperature-tolerance gene

With the chromosomal DNA library of Acetobacter altoacetigenes MH-24 obtained in the above-described manner, Acetobacter aceti No. 1023 strain (FERM BP-2287) that generally

can only grow at a growth temperature of up to around 37°C on an agar medium was transformed. Subsequently, the transformed Acetobacter aceti No. 1023 strain was cultured for 4 days at 38°C on YPG agar medium to which 100 μg/ml of ampicillin was added.

Then, the formed colony was inoculated into YPG medium containing 100 μg/ml of ampicillin and cultured, and plasmids were recovered from the obtained bacterial cells. As a result, a plasmid, in which a SalI-KpnI fragment of approximately 1.6 kbp as shown in Fig. 1 was cloned, could be recovered, and this plasmid was named pG1.

In this way, the DNA fragment for enhancing temperature tolerance, which enables Acetobacter aceti No. 1023 strain that generally can only grow at up to around 37°C on an agar medium to grow even at 38°C was obtained.

(3) Determination of the nucleotide sequence of the cloned DNA fragment and the amino acid sequence

The cloned SalI-KpnI fragment described above was inserted into the SalI-KpnI cleavage site of pUC19, and the nucleotide sequence of the fragment was determined by Sanger's dideoxy chain termination method. Determination of the nucleotide sequence was conducted in all domains of the both strands of DNA, and it was also conducted in such a manner that the cleavage sites were overlapped.

As a result, the nucleotide sequence shown in SEQ. ID

No. 1 in the sequence listing was determined. In nucleotides 73 to 1251 in the nucleotide sequence shown in SEQ. ID No. 1 in the sequence listing, the presence of the open reading frame (ORF) encoding the amino acid sequence consisting of 393 amino acids shown in SEQ. ID No. 2 in the sequence listing was confirmed.

(Example 2) Enhancement of temperature tolerance in transformant transformed with temperature-tolerance gene derived from Gluconacetobacter entanii

(1) Transformation of Acetobacter aceti

The gene for enhancing temperature tolerance derived from Acetobacter altoacetigenes MH-24 strain (FERM BP-491) cloned as in Example 1 described above was amplified by the PCR method using KOD-Plus- (manufactured by TOYOBO Co., LTD). Then, pGCS was constructed by inserting the amplified fragment into the restriction enzyme SmaI cleavage site of the acetic acid bacteria-Escherichia coli shuttle vector pMV24 (see non-patent literature 3, for instance). The outline of the amplified fragment inserted into pGCS is shown in Fig. 1. This amplified fragment is included in the SalI-KpnI fragment (gene for improving temperature tolerance: the nucleotide sequence thereof is shown in SEQ. ID No. 1), and includes a part of the upstream and the downstream regions of the coding region (ORF) of the nucleotides 73 to 1251.

The PCR method was performed as follows; i.e. the PCR method was performed using genomic DNA derived from the above-mentioned acetic acid bacteria as a template and using primer 1 (the nucleotide sequence thereof is shown in SEQ. ID No. 3 (Fig. 5)) and primer 2 (the nucleotide sequence thereof is shown in SEQ. ID No. 4 (Fig. 6)) as a primer in the following condition.

(PCR condition)

The PCR method was performed for 30 cycles with one cycle consisting of 94°C for 15 sec, 60°C for 30 sec and 68°C for 2 min.

Acetobacter aceti No. 1023 strain (FERM BP-2287) was transformed with this pGCS by the electroporation method (see non-patent literature 6, for instance). The transformant was selected by culturing it on YPG agar medium, to which 100 μg/ml of ampicillin was added, at a culture temperature of 38°C.

It was confirmed that the transformant with ampicillin resistance grown on the selective medium retained the plasmid having the temperature-tolerance gene (GCS gene) by extracting the plasmid from the transformant in the conventional manner and analyzing it.

(Example 3) Acetic acid fermentation test of the transformant transformed with temperature-tolerance gene derived from Gluconacetobacter entanii

(1) Temperature tolerance of the transformant

The ampicillin-resistant transformant having the plasmid pGCS obtained in Example 2 was compared with the original strain of Acetobacter aceti No. 1023 into which only the shuttle vector pMV24 was introduced with regard to the growth in YPG medium when the culture temperature was changed.

Specifically, aeration-agitation culture was carried out in 1 L of YPG medium containing 1% acetic acid, 4% ethanol and 100 μg/ml of ampicillin using a 2-L mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-2A) at 400 rpm and 0.2 vvm, and the concentration of acetic acid in the culture medium and the growth for the transformant (by measuring the absorbance at 660 nm) was compared to those for the original strain. The culture temperature was 30°C at first, then fermentation was performed at 33°C until the concentration of acetic acid reached about 3%. Then, the temperature was further raised to 36°C and fermentation was performed until the concentration of acetic acid reached 3%. Thereafter, the temperature was raised in 2°C increments, and acetic acid fermentation was performed. When the concentration of acetic acid reached 3%, the culture medium was withdrawn except for about 100 ml of the culture medium, which was left in the mini-jar fermenter. To the remaining 100 ml of the culture medium, 900 ml of YPG medium was added in such a manner that the final concentrations of acetic acid, ethanol and ampicillin were 1%, 4% and 100 μg/ml,

respectively, and the temperature was changed in the above-described manner, and acetic acid fermentation was restarted.

As a result, as shown in Fig. 2, while in the original strain of Acetobacter aceti No. 1023 strain, growth of bacteria and acetic acid fermentation could confirmed only up to 37°C, in the transformant, growth of bacteria and acetic acid fermentation were possible even at 38°C, and moreover, the result in which the growth was confirmed even at 40°C was obtained, whereby the function of enhancing temperature tolerance of GCS gene was confirmed.

(Example 4) Enhancement of temperature tolerance in the transformant transformed with temperature-tolerance gene derived from Gluconacetobacter entanii

(1) Transformation of Acetobacter aceti

The gene for enhancing temperature tolerance derived from Acetobacter altoacetigenes MH-24 strain (FERM BP-491) cloned as in Example 1 described above was amplified by the PCR method using KOD-Plus- (manufactured by TOYOBO Co., LTD.). Then, pGCS1 was constructed by inserting the acetic acid bacteria-Escherichia coli shuttle vector pGI18 (see non-patent literature 2, for instance) into the restriction enzyme SmaI cleavage site. The outline of the amplified fragment inserted into pGCS1 is shown in Fig. 1. This amplified

fragment is included in the SalI-KpnI fragment (gene for improving temperature tolerance: the nucleotide sequence thereof is shown in SEQ. ID No. 1), and includes a part of the upstream and the downstream regions of the coding region (ORF) of the nucleotides 73 to 1251.

The PCR method was performed as follows; i.e. the PCR method was performed using the above-mentioned genomic DNA derived from acetic acid bacteria as a template and using primer 1 (the nucleotide sequence thereof is shown in SEQ. ID No. 3 (Fig. 5)) and primer 2 (the nucleotide sequence thereof is shown in SEQ. ID No. 4 (Fig. 6)) as a primer in the following condition.

(PCR condition)

The PCR method was performed for 30 cycles with one cycle consisting of 94°C for 15 sec, 60°C for 30 sec and 68°C for 2 min.

Acetobacter aceti No. 1023 strain (FERM BP-2287) was transformed with this pGCS1 by the electroporation method (see non-patent literature 6, for instance). The transformant was selected by culturing it on YPG agar medium, to which 100 μg/ml of ampicillin was added, at a culture temperature of 38°C.

It was confirmed that the transformant with ampicillin resistance grown on the selective medium retained the plasmid having the GCS gene by extracting the plasmid from the transformant in the conventional manner and analyzing it.

The vector pGI18 for acetic acid bacteria was constructed

by the procedure shown in Fig. 8.

This vector pGI18 was constructed from the plasmids pGI1 and pUC18. First, the plasmid pGI1 was constructed.

That is, Acetobacter altoacetigenes MH-24 strain (FERM BP-491), which is one strain of Gluconacetobacter entanii, was cultured with shaking at 30°C for 240 h to 336 h in YPG medium (containing 3% glucose, 0.5% yeast extract and 0.2% polypeptone) to which 6% acetic acid and 4% ethanol were added. The obtained bacterial cells were lysated with sodium hydroxide or sodium dodecyl sulfate, then treated with phenol and further with ethanol, whereby the plasmid DNA was purified.

The obtained plasmid DNA was digested with various restriction enzymes (manufactured by Takara Bio Inc.) (at 37°C and an enzyme concentration of 1 unit/ml), and the base-pair size of the obtained DNA fragments was determined by agarose electrophoresis.

The obtained plasmid DNA is a plasmid consisting of cyclic double stranded DNA having three HincII recognition sites and one SfiI recognition site, and the total size of the plasmid was about 3100 base pairs (bp). In addition, it had no recognition site for EcoRI, SacI, KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI and HindIII. The obtained plasmid DNA was named plasmid pGI1 and was used in construction of the vector pGI18 (Fig. 8).

The plasmid pGI1 obtained above was amplified by the PCR

method using KOD-Plus- (manufactured by TOYOBO Co., LTD.) and digested with AatII.

The PCR method was performed as follows; i.e. the PCR method was performed using the plasmid pGI1 prepared in Example 1 as a template and using primer A and primer B having an AatII recognition site as a primer in the following condition. The nucleotide sequences of the primer A and the primer B are as shown in SEQ. ID No. 6 (Fig. 12) and in SEQ. ID No. 7 (Fig. 13), respectively.

The PCR condition was 30 cycles with one cycle consisting of 94°C for 30 sec, 60°C for 30 sec and 68°C for 3 min.

On the other hand, pUC18 harboring an ampicillin (Amp)-resistance gene (manufactured by Takara Bio Inc.: 2686 bp) was digested with AatII (at 37°C and an enzyme concentration of 1 unit/ml) and ligated to the plasmid pGI1 obtained above with T_4 DNA ligase. Then, Escherichia coli JM109 strain (manufactured by Takara Bio Inc.) was transformed in the conventional manner by using the reaction solution after ligation, and the transformant with Amp resistance was obtained by selection on a plate of LB medium (10 g of triptone, 5 g of yeast extract and 5 g of NaCl/L) containing 100 μg/ml of sodium ampicillin. From the obtained transformant, the plasmid was prepared, and the restriction enzyme cleavage pattern thereof was analyzed. In Fig. 8, the restriction enzyme map of the obtained plasmid is shown. In Fig. 8, "AatII"

and "SfiI" indicate the restriction enzyme recognition sites. In addition, MCS indicates a multicloning site, Amp^r indicates an ampicillin-resistance gene site, and a number in the parenthesis indicates the number of the nucleotide components represented by the bp unit. Further, "pUC18", "pGI1" and "pGI18" in the middle indicate the names of the plasmids, and "2.7 kbp", "3.1 kbp" and "5.8 kbp" indicate the total base numbers of the plasmids.

As is clear from Fig. 8, the obtained plasmid contained both pUC18 and pGI1, and the total size thereof was about 5800 base pairs (5.8 kbp). This plasmid was named vector pGI18 for acetic acid bacteria.

The nucleotide sequence of this vector pGI18 is shown in SEQ. ID No. 5 (Fig. 9, Fig. 10 and Fig. 11).

(2) Temperature tolerance of the transformant

The ampicillin-resistant transformant having the plasmid pGCS1 obtained in the above-described manner was compared with the original strain of Acetobacter aceti No. 1023 into which only the shuttle vector pGI18 was introduced with regard to the growth in YPG medium when the culture temperature was changed.

Specifically, aeration-agitation culture was carried out in 1 L of YPG medium containing 1% acetic acid, 4% ethanol and 100 μg/ml of ampicillin using a 2-L mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-2A) at 400 rpm and 0.2 vvm,

and the concentration of acetic acid in the culture medium and the growth for the transformant (by measuring the absorbance at 660 nm) was compared to those for the original strain. The culture temperature was 30°C at first, then fermentation was performed at 33°C until the concentration of acetic acid reached about 3%. Then, the temperature was further raised to 36°C and fermentation was performed until the concentration of acetic acid reached 3%. Thereafter, the temperature was raised in 2°C increments, and acetic acid fermentation was performed. When the concentration of acetic acid reached 3%, the culture medium was withdrawn except for about 100 ml of the culture medium, which was left in the mini-jar fermenter. To the remaining 100 ml of the culture medium, 900 ml of YPG medium was added in such a manner that the final concentrations of acetic acid, ethanol and ampicillin were 1%, 4% and 100 μg/ml, respectively, and the temperature was changed in the above-described manner, and acetic acid fermentation was restarted.

As a result, as shown in Fig. 3, while in the original strain of Acetobacter aceti No. 1023 strain, growth of bacteria and acetic acid fermentation could confirmed only up to 37°C, in the transformant, growth of bacteria and acetic acid fermentation were possible even at 38°C, and moreover, the result in which the growth was confirmed even at 40°C was obtained, whereby the function of enhancing temperature

tolerance of GCS gene was confirmed.

(Example 5) Acetic acid fermentation test of the transformant transformed with temperature-tolerance gene derived from Gluconacetobacter entanii

(1) Transformation of Acetobacter altoacetigenes

Acetobacter altoacetigenes MH-24 strain (FERM BP-491), which is one strain of Gluconacetobacter entanii, was transformed with the plasmid pGCS1 obtained as in (Example 4) by the electroporation method (see non-patent literature 6, for instance). The transformant was selected with YPG agar medium containing 0.55% agar to which 100 μg/ml of ampicillin, 4% acetic acid and 4% ethanol were added.

It was confirmed that the transformant with ampicillin resistance grown on the selective medium retained the plasmid having the gene for enhancing temperature tolerance by extracting the plasmid from the transformant in the conventional manner and analyzing it.

(2) Acetic acid fermentation test

The ampicillin-resistant transformant having the plasmid pGCS1 obtained in (1) was compared with the original strain of Acetobacter altoacetigenes MH-24 strain into which only the shuttle vector pGI18 was introduced with regard to the acetic acid fermentation ability.

Specifically, aeration-agitation culture was carried

out in 2.5 L of a raw material medium (7% acetic acid, 3% ethanol, 0.2% yeast extract and 0.2% glucose) containing 100 μg/ml of ampicillin using a 5-L mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-5A) at 30°C, 500 rpm and 0.20 vvm. At the stage when apparent growth of bacteria was confirmed and the concentration of remaining ethanol reached 2%, a solution containing ethanol (1% acetic acid, 50% ethanol, 0.2% yeast extract and 0.2% glucose) was fed, whereby the concentration of ethanol in the fermentation liquid was maintained at 2%. By this method of performing acetic acid fermentation, acetic acid fermentation ability of the transformant was compared to that of the original strain. The result is summarized in Table 1.

(Table 1)

	Final acetic acid concentration (%)	Specific growth rate (OD 660/hr)	Production rate (%/hr)
Original strain	15.6	0.0061	0.31
Transformant	17.2	0.0061	0.26

From the result of Table 1, it was confirmed that the transformant was remarkably superior in the final acetic acid concentration.

(3) Transformation of Acetobacter aceti subsp. xylinum

Acetobacter aceti subsp. xylinum IFO3288 strain, which is one strain of Acetobacter aceti subsp. xylinum, was transformed with the plasmid pGCS1 obtained as in (Example 4) by the electroporation method (see non-patent literature 6,

for instance). The transformant was selected with YPG agar medium to which 100 μg/ml of ampicillin was added.

It was confirmed that the transformant with ampicillin resistance grown on the selective medium retained the plasmid having the gene for enhancing temperature tolerance by extracting the plasmid from the transformant in the conventional manner and analyzing it.

(4) Acetic acid fermentation test

The ampicillin-resistant transformant having the plasmid pGCS1 obtained in (3) was compared with the original strain of Acetobacter aceti subsp. xylinum IFO3288 strain into which only the shuttle vector pGI18 was introduced with regard to the acetic acid fermentation ability.

Specifically, aeration-agitation culture was carried out using a raw material medium prepared by mixing 17.9% saccharified rice solution, 3.2% fermented moromi, 7.8% alcohol for brewing and 71.1% water at such a ratio (concentration of alcohol: 7.8%, concentration of acetic acid: 0.26%) in a 5-L mini-jar fermenter (Mitsuwa Rikagaku Kogyo Co.; KMJ-5A) at 30°C, 500 rpm and 0.20 vvm, and continuous fermentation at an acetic acid concentration of 7.2% was performed. The rates of adding the raw material medium in the continuous fermentation at an acetic acid concentration of 7.2% were compared, and the result is shown in Table 2. In addition, the acetic acid fermentation abilities were compared when the

raw material medium addition rate for the transformant was conformed to the raw material medium addition rate for the original strain in the continuous fermentation at an acetic acid concentration of 7.2%. The result is shown in Table 3.

(Table 2)

	Acetic acid concentration (%)	OD 660	Raw material medium addition rate (g/hr)
Original strain	7.17	0.538	84.7
Transformant	7.26	0.612	96.3

(Table 3)

	Acetic acid concentration (%)	OD 660	Raw material medium addition rate (g/hr)
Original strain	7.24	0.502	82.3
Transformant	7.61	0.437	82.0

From the results of Table 2 and Table 3, it was confirmed that also in the continuous acetic acid fermentation, the transformant was remarkably superior in the productivity (raw material medium addition rate) and the concentration of produced acetic acid.

Industrial Applicability

According to the present invention, a novel gene participating in temperature tolerance can be provided, further a bred strain capable of highly efficient production vinegar under a higher temperature condition can be obtained by using the gene, and a method of highly efficient production vinegar under a higher temperature condition using the bred

strain can be provided.